

## MiniReview

# The VirE2 protein of *Agrobacterium tumefaciens*: the Yin and Yang of T-DNA transfer

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**Abstract**

*Agrobacterium tumefaciens* has evolved a unique mechanism to solve the problem of transferring DNA across five bilayers; the inner and outer membranes of the bacterium, the plasma membrane of the plant cell and the double membrane formed by the nuclear envelope. The two first and two last seem to be mediated by, respectively, the type IV secretion system in *Agrobacterium* and the nuclear pore complex in the plant cell, but the mechanism by which the transferred DNA (T-DNA) crosses the plant membrane still remains a mystery. New biophysical experiments suggest that, in addition to its established role as a single-stranded DNA (ssDNA)-binding protein, the VirE2 protein forms a channel in the plant membrane allowing the passage of the T-DNA into the cell. Such a role would be reminiscent of translocator molecules secreted by the type III secretion system of pathogenic bacteria and inserting into the host eukaryotic plasma membrane. The implications for the structure of the protein, its regulation and role in vivo are discussed.

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**Keywords:** *Agrobacterium*; VirE2; DNA channel

**1. Introduction**

The transfer of DNA to and between cells is not only an essential process in cell division and bacterial conjugation, but also in pathogenesis-related processes like those that occur in the crown gall disease caused by *Agrobacterium tumefaciens*. This Gram-negative bacterium has evolved a unique mechanism to transfer a segment of DNA (T-DNA, for transferred DNA) from its tumor inducing plasmid (pTi) to the cytoplasm of plant cells (Fig. 1). The T-DNA is then transferred to the nucleus of the host cell where it integrates into the chromosomal DNA. The expression of the genes located on the T-DNA leads to uncontrolled growth of plant cells, hence the name ‘crown gall’ given to the disease.

The single-stranded DNA (ssDNA) transfer needs the activity of several pTi-encoded proteins, the virulence (Vir) proteins. The 11 VirB proteins and VirD4 are thought to form a channel spanning the inner and outer membrane of

*Agrobacterium*, hence allowing the passage of the T-DNA out of the bacterium. This multiprotein complex forms the *Agrobacterium* type IV secretion system [1]. Upon induction of the virulence genes by chemical signals emanating from wounded plant cells, a ‘virulence pilus’ is formed on the surface of the bacterium. Its exact role in T-DNA transfer has not been determined yet [2]. However, it has been proposed to form a continuum with the channel formed by the VirB proteins and to puncture the plant plasma membrane to deliver the T-DNA and the effector proteins into the cytoplasm [3]. The T-DNA per se is piloted by another Vir protein, VirD2, which is attached covalently to the 5′ end of the T-DNA. The presence of VirD2 is essential for initiation of nuclear import of the T-DNA, possibly through its interaction with importins [4]. Continued import of the T-DNA–VirD2 complex into the nucleus seems to require yet another Vir protein, VirE2 [5].

**2. The functions of the VirE2 protein**

More than 15 years ago, the VirE2 protein was shown to bind ssDNA [6]. This binding is not sequence-specific

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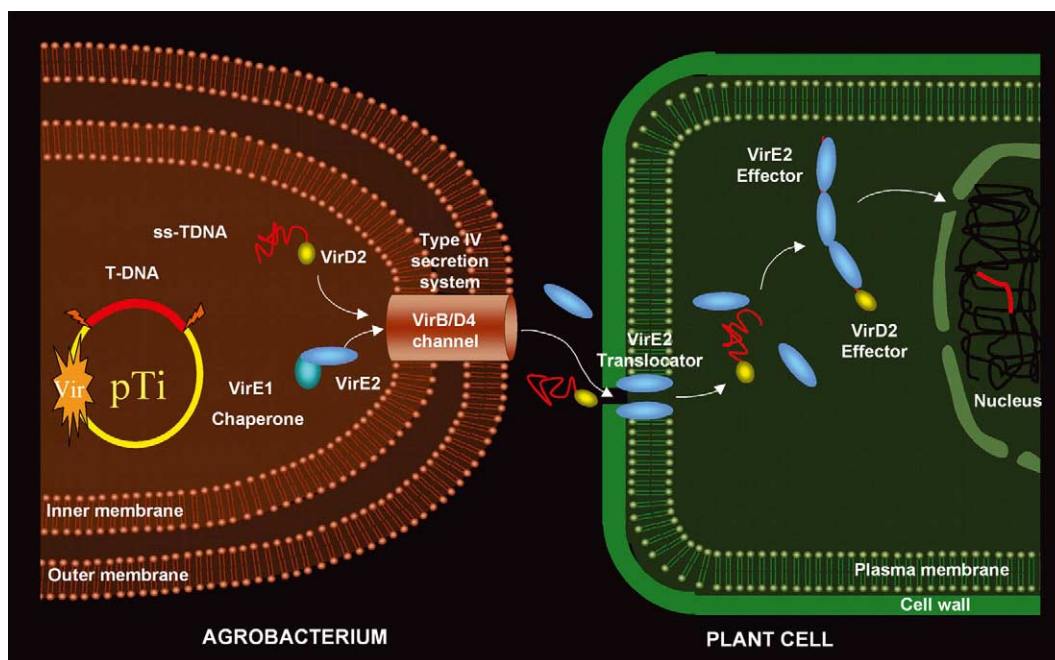


Fig. 1. Hypothetical model for T-DNA transfer from an agrobacterial cell into a plant cell. In the bacterial cytoplasm, the VirE1 chaperone prevents binding of VirE2 to ssDNA, and possibly prevents VirE2-dependent channel formation in the bacterial membrane. VirE2 is exported through the type IV VirB–VirD4 channel and subsequently inserts into the plant plasma membrane, allowing the transport of the ssDNA–VirD2 complex into the plant cytoplasm. Once in the plant cytoplasm, VirE2 molecules coat the complex, permitting its transfer to the nucleus. For simplicity, the pilus and its involvement in T-DNA transfer are omitted from the scheme (modified after fig. 4 from [10]).

and protects the T-DNA from nucleases [7]. Upon binding of the VirE2 protein, the VirE2/ssDNA complex coils into a regular telephone cord structure [8]. This highly ordered structure might be extended *in vivo* to facilitate transport through the nucleopore complex [5].

The VirE2 protein is by far the largest known prokaryotic ssDNA-binding protein; with 60 kDa it is twice the size of RecA or SSB. The fact that pathogens usually pack their virulence proteins with several functions led to the hypothesis that the VirE2 protein may have an additional function. Several experimental results now show this to be the case. As the VirE2 protein had been found in the membrane fractions of *Agrobacterium* [9], the possibility that the VirE2 protein could associate with the membrane was investigated by biophysical means [10]. First, to determine whether VirE2 interacts with lipids, Langmuir-trough experiments were performed. In this assay a monolayer of lipids at an air/buffer interface is compressed in absence and presence of the tested protein. If the studied protein interacts with lipids, the compression isotherm will be shifted towards a larger effective area per lipid molecule. This shift can be explained either by insertion of the protein into the monolayer or by an adsorption process modifying the lipid organization at the air–water interface. The addition of the VirE2 protein did induce a shift, showing that the VirE2 protein is somehow interacting with the lipid monolayer. Second, since this technique did not determine if the VirE2 protein is able to integrate in a lipid bilayer, black lipid membrane assays were performed. In this method, a lipid bilayer is assembled on a

small diaphragm separating two compartments forming a non-conductive bilayer. Upon addition of a membrane protein forming a channel and application of a voltage, ions can pass from one compartment to the other. This flow of ions can be measured as a current and allows the determination of the conductance of the channel under study. Upon addition of the VirE2 protein and application of a voltage of 100 mV, large conductance jumps were measured, revealing that the VirE2 protein indeed had inserted into the lipid bilayer and had formed a channel. The insertion in the membrane of a living organism (bacteria or eukaryotic cells) of such a channel (bacteria or eukaryotic cells) would be deleterious, disrupting the membrane potential and allowing leakage of essential metabolites. To avoid this counterproductive effect many channels are regulated by the membrane potential and close at a defined voltage. To test if VirE2 is a voltage-gated channel, we subjected the VirE2 channel inserted into the lipid bilayer to increasing voltage. Until  $-120$  mV, a conductance could be measured, but at  $-120$  mV, no conductance could be detected, indicating that the channel was closed at a potential close to the one occurring in plant cells ( $-100$  to  $-250$  mV, [11,12]).

The VirE2 channel was found to preferentially allow the passage of negatively charged molecules: it was anion selective. Since the T-DNA is a large, negatively charged molecule, the possibility that the VirE2 channel could transport ssDNA was investigated by a vesicle swelling assay and a radioactive DNA transport assay. Both require lipid vesicles containing the VirE2 protein. The

vesicle swelling assay resulted in a decrease of light scattering upon incubation of the VirE2 proteoliposomes with ssDNA, indicating that the VirE2 protein containing vesicles were swelling due to uptake of water-accompanied ssDNA. The radioactive DNA transport assay, where VirE2 proteoliposomes were incubated with radioactive ssDNA, resulted in accumulation of radioactive ssDNA inside the VirE2 proteoliposomes, but not in liposomes lacking VirE2. These results clearly demonstrated that the VirE2 protein forms a channel able to transport ssDNA across a lipid bilayer.

### 3. Bacterial toxins as a model for the transition from soluble to membrane-embedded proteins

These results were surprising because studies in the preceding 15 years failed to indicate the possibility that VirE2 could form a channel. The dual nature of the VirE2 protein is unique: on the one hand it must be soluble and must be able to bind ssDNA, on the other hand it has to integrate in the hydrophobic environment of the lipid bilayer and to form a DNA channel. We call this at first sight unsurmountable challenge the Yin and Yang property of the VirE2 protein. In the membrane, VirE2 is expected to retain some affinity for DNA as the channel is selective for anions and the affinity for ssDNA is high. However, by definition, this interaction has to be transient to allow passage of the DNA. The question then is, how a

single protein can possess such apparently conflicting qualities, especially since analysis of the VirE2 sequence does not identify any  $\alpha$ -helical transmembrane domains?

To answer this, a glance at other 'Yin and Yang' bacterial proteins also involved in pathogenesis may help. One such a protein is the  $\alpha$ -hemolysin of *Staphylococcus aureus*. This protein is secreted from the bacterium and inserts in the membrane of the target eukaryotic cell. Here it forms a large channel that will eventually kill the cell by bilayer permeation to ions, water and small solutes [13]. Thus, like VirE2, the  $\alpha$ -hemolysin protein has to be able to adjust to the hydrophilic environment it encounters when it is secreted, as well as the hydrophobic environment of the lipid bilayer. The structure of the membrane-bound form of the  $\alpha$ -hemolysin has been solved, but not the structure in the soluble form. However, the structure of a homologous toxin, LukF from *S. aureus* has been solved in its soluble state. The comparison of the structure of the soluble state (LukF [14]) and the structure of the membrane form of  $\alpha$ -hemolysin [13] indicates how this structural challenge may be met [15]. The soluble LukF is rich in  $\beta$ -strands and exists as a monomer (Fig. 2a). In contrast, the membrane form of  $\alpha$ -hemolysin is a heptamer (Fig. 2b). Each monomer of this heptamer provides a  $\beta$ -strand hairpin to form a 14-stranded  $\beta$ -barrel that crosses the membrane. The  $\beta$ -barrel structure was first discovered in bacterial porins [16] and is one of the two basic structural forms adopted by membrane proteins. In the soluble state of LukF, the  $\beta$ -strand hairpin that forms

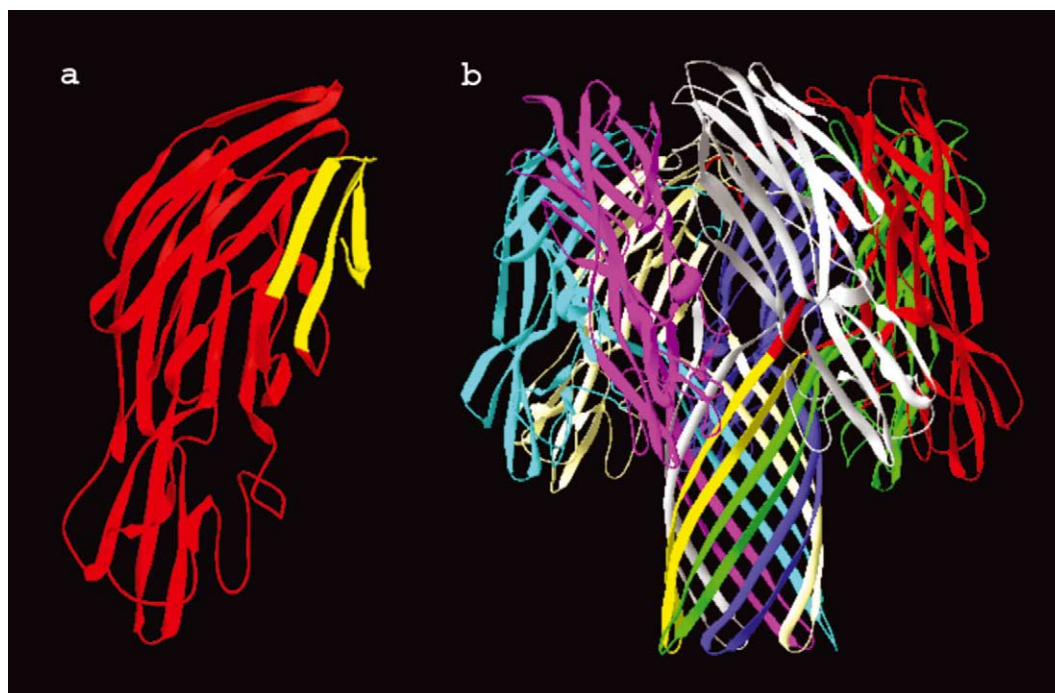


Fig. 2. Ribbon drawings of (a) the *S. aureus* soluble LukF monomer and of (b) the *S. aureus* membrane-bound  $\alpha$ -hemolysin heptamer, perpendicular to the seven-fold axis. Each subunit is displayed in a different color. a: The LukF monomer consists mainly of  $\beta$ -strands. The  $\beta$ -sheet undergoing a conformational change upon insertion in the membrane is highlighted in yellow. b: This yellow region forms a long  $\beta$ -strand hairpin in the heptamer. Upon heptamerization, the  $\beta$ -strand hairpins associate to form a 14-stranded  $\beta$ -barrel, a fold able to integrate in the membrane and form a large channel.

the barrel in the membrane form of the  $\alpha$ -hemolysin is folded back on the monomer [14]. It is therefore protected from the hydrophilic environment, thus possibly explaining the solubility of this protein.

From the above, the key properties allowing  $\alpha$ -hemolysin protein to be both soluble and to be able to insert into the membrane are: (1) the ability to oligomerize, (2) to possess a rich content of  $\beta$ -strands allowing the formation of a  $\beta$ -barrel upon oligomerization, and (3) the fact that only 30 amino acids (10% of the total protein) insert in the membrane. Does the VirE2 protein possess any of these qualities? Early biochemical characterization of the protein showed that the VirE2 protein is also able to oligomerize, possibly into a tetramer [17]. More recently, preliminary circular dichroism results indicate that VirE2 is a protein rich in  $\beta$ -strands (M. Duckely, unpublished result). So, two key features that meet the requirement for an  $\alpha$ -hemolysin-like channel seem to be fulfilled. The analysis of mutants impaired in oligomer formation should help to determine the importance of the oligomer in forming the channel. Analysis of VirE2 molecules integrated in the lipid bilayer should allow the portion of the protein located in the membrane to be defined.

#### 4. In vivo relevance of the VirE2 channel

Our biophysical results suggest that the purified VirE2 protein is able to form large anion selective channels. However, these are in vitro results and so far the in vivo existence, let alone the importance of the VirE2 channel in T-DNA transfer has not yet been demonstrated. In *Agrobacterium*, VirE2 is associated with its specific 'chaperone' (as defined in the type III secretion systems), VirE1. This interaction is needed for export of VirE2 from *Agrobacterium* [18]. Recent structural analysis of four different chaperones of the TTSS (type III secretion systems) seems to show that only a chaperone-effector complex can allow the effector to be in a secretion-competent conformation [19]. The structural analysis also implied that unfolding of the effector molecules is probably limited to the chaperone-binding domain. This would mean that a secretion-competent conformation of the effector may be limited to a particular domain of the translocated protein, leaving the rest of the effectors in their respective fully active conformation. In the light of these new results, the ability of the VirE2–VirE1 complex to interact with lipids and to bind ssDNA should be investigated in vitro.

During or after secretion from *Agrobacterium*, the VirE2 protein dissociates from its VirE1 chaperones. It remains to be determined when precisely this occurs and what triggers the dissociation. In other secretion systems, such as the type III secretion system of *Yersinia pestis*, the chaperone is not secreted with its cognate protein [20]. The mechanism of VirE2 channel assembly in the plant membrane is also unknown. Several proteins that form homo-

multimeric channels including the above-mentioned  $\alpha$ -hemolysin have been shown to interact with a target cell receptor to increase the monomeric protein concentration. This would favor the insertion in the membrane and the formation of a multimeric channel [15]. However, a receptor that interacts with VirE2 has not yet been identified.

Another important issue is the localization of the VirE2 protein in plants transgenic for VirE2. In 1992, Citovsky et al. reported the surprising finding that a *virE2* defective *Agrobacterium*, normally defective in T-DNA transfer, could be rescued if these agrobacteria were infecting tobacco plants transgenic for VirE2 [21]. This experiment showed that the VirE2 protein was needed in late infection steps occurring in the plant. In this same paper, the localization of a GUS–VirE2 fusion was investigated. The fusion protein localized in the nucleus of plant cells and this localization was dependent on the presence of NLSs (nuclear localization signals) on VirE2. These results are in apparent conflict with our proposed role of VirE2 as an entry gate for T-DNA through the plasma membrane of plant cells. Indeed, such a role would imply a localization of VirE2 at the plasma membrane. However, the GUS–VirE2 fusion protein used in the study mentioned above may be unable to insert into the membrane. Indeed, more recent studies showed that VirE2 can tolerate a peptide insertion near its N-terminus, but fusions to heterologous proteins at either terminus render the VirE2 protein inactive [22]. These findings support a model in which VirE2 oligomerization is dependent on proper folding of the full-length protein. As channel formation might rely on oligomerization of VirE2, the localization of the GUS–VirE2 fusion in the nucleus might be explained by defective oligomerization of this fusion protein. Moreover, the GUS–VirE2 fusion was not tested for restoration to virulence of a VirE2 defective agrobacterial strain. In light of the in vitro channel formation of VirE2, a localization of VirE2 expressed in plant cells has to be carefully reinvestigated.

#### 5. Regulation of channel opening

*Agrobacterium* pathogenesis, contrary to pathogenesis by most other agents that kill their host, requires that the transformed plant cell remains alive and healthy to be able to divide, form the crown gall and produce the opines that only *Agrobacterium* can metabolize. Hence, compared to other bacterial toxins secreted by bacteria to kill eukaryotic cells, the action of the *Agrobacterium* virulence proteins, including the VirE2 protein has to be subtle. This implies some regulation mechanisms, which may involve the activity of the T-DNA pilot, VirD2. The VirE2 channel inserted in the plant plasma membrane will most likely be closed, due to the potential of plant membranes (–100 to –250 mV [11,12]). If the T-DNA pilot were able to open the VirE2 channel, it would be



the perfect key to unlock the gate of the channel only when and where it is needed. This scenario would imply that the VirD2 protein, covalently bound to ssDNA, is able to pass the VirE2 channel. Such a passage of a presumably globular protein is far from trivial from a structural point of view.

## 6. Energy requirements of DNA transfer

Radioactive DNA transport assays described above have shown the passage of oligonucleotides through the VirE2 channel [10]. This transport is likely driven by the DNA concentration gradient. However, in the *in vivo* context, the T-DNA has to be transported from the extracellular medium to the interior of the plant cell and no gradient of DNA is present. Energy is likely to be needed, but where does it come from? In another ssDNA transfer system, the bacterial conjugation of the *Escherichia coli* R388 system, the protein TrwB is thought to form a channel through which ssDNA can pass from one bacterial cell to another. The three-dimensional (3D) structure of the soluble form has been reported and showed that TrwB forms hexamers and possesses a nucleotide-binding domain. Nucleotide hydrolysis possibly provides the energy to transport the ssDNA from one bacterium to another [23]. The VirE2 protein does not possess a nucleotide-binding domain, but the VirB4 and VirB11 proteins of the agrobacterial type IV secretion apparatus are the proposed traffic ATPases. These proteins may provide the energy for translocation of the T-DNA if there is a continuum between the VirB/VirD4 apparatus and the VirE2 channel. This would imply interactions between the VirE2 channel and the VirB1–11/VirD4 type IV secretion system of *Agrobacterium*, a configuration that would protect the T-DNA, allow a high efficiency of transfer and provide the needed energy. A similar mechanism has been proposed for the secretion of effector proteins in *Yersinia* pathogenesis [24].

Another interesting hypothesis gained from the X-ray structure of  $\Delta 70$ TrwB concerns the aqueous face of this channel.  $\Delta 70$ TrwB corresponds to a truncation of 70 amino acids at the N-terminus, a region corresponding to two putative transmembrane helices. Their removal allowed the purification and crystallization of a soluble form of the protein, but the full understanding of the ssDNA passage through the bacterial lipid bilayer still awaits the resolution of the structure of the full-length protein. In the region where the transmembrane  $\alpha$ -helices can be modelled, 12  $\beta$ -strands are localized in close proximity to one another. This led to the hypothesis that the aqueous face of the channel formed by TrwB is composed of a 12-stranded  $\beta$ -barrel. Hence, the  $\beta$ -barrel fold is a structural solution for both the delicate transition from soluble to membrane bound, and possibly for the passage of ssDNA through membranes.

## 7. Conclusions and perspective

Apart from having evolved a unique DNA transfer mechanism, the strategies *Agrobacterium* has developed are similar to those employed by other pathogens. To secrete the effector proteins, both type III and type IV secretion machines form a multiprotein channel spanning the inner and outer membrane of the bacterium and extensively use chaperones to keep the effectors in an export-competent state. Both systems export the effectors, the roles of which are in the target eukaryotic cell. The first effectors are the translocators, which target the eukaryotic cell membrane, allowing the passage of the other effector molecules, which function inside the eukaryotic cell. If indeed the recently discovered VirE2 channel is formed in the plant membrane, it will be reminiscent of other translocator proteins such as the YopB/D channel formed in the human cell membrane by *Yersinia*. Thus, the fact that VirE2 would act as a translocator would constitute yet another similarity between the type III and type IV secretion systems. VirE2 would then be the first identified translocator of a type IV secretion system.

The discovery that the VirE2 channel is able to transfer ssDNA at least *in vitro* is exciting and invites further experiments to understand the channel gating and the possibly regulated opening mechanism. One of the puzzling aspects of the function of the VirE2 protein as a ssDNA-binding protein and as a ssDNA channel is the need on the one hand for a stable ssDNA-binding activity of soluble VirE2 protein and, on the other hand, of transient ssDNA binding for transport of the ssDNA through the VirE2 channel. Only the atomic structure of the soluble VirE2 protein bound to ssDNA and of the VirE2 channel interacting with ssDNA will resolve this fascinating structural aspect of T-DNA transfer. The deeper understanding of this transfer process might then allow the use of the VirE2 channel as a more general tool for DNA transfer into eukaryotic cells.

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